

A mutation in the Zn-finger of the GAL4 homolog LAC9 results in glucose repression of its target genes

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ABSTRACT

The transcriptional activator LAC9, a GAL4 homolog of *Kluyveromyces lactis* which mediates lactose and galactose-dependent activation of genes involved in the utilization of these sugars can also confer glucose repression to those genes. Here we report on the isolation and characterization of *LAC9-2*, an allele which encodes a glucose-sensitive activator in contrast to the one previously cloned. A single amino acid exchange of leu-104 to tryptophan is responsible for the glucose-insensitive phenotype. The mutation is located within the Zn-finger-like DNA binding domain which is highly conserved between LAC9 and GAL4. Glucose repression is also eliminated by duplication of the *LAC9-2* allele. The data indicate that LAC9 is a limiting factor for β-galactosidase gene expression under all growth conditions and that glucose reduces the activity of the activator.

INTRODUCTION

When yeast cells are grown in glucose-containing media the expression of a large number of genes including those involved in disaccharide utilization is shut off or greatly reduced. This phenomenon has been termed glucose repression or catabolite repression after an analogous situation in *Escherichia coli* (1) but the molecular mechanism in yeast is clearly different (2,3). A large number of mutants have been isolated in *Saccharomyces cerevisiae* which affect this process (see (4,5) for reviews) but the intracellular signal induced by glucose and its transmission to the transcriptional machinery are points which are still not understood. Only for the last step in the signal transduction chain, the regulation of transcription in response to glucose, are molecular details beginning to emerge for a few genes, revealing a large complexity in the mechanisms.

For example, glucose repression of the *ADH2* gene encoding alcohol dehydrogenase involves phosphorylation of the transcription factor ADR1 (6,7). At least part of this phosphorylation depends on a cAMP-dependent protein kinase. Thus, cAMP stimulates repression in contrast to the situation in *E. coli* in which catabolite repression is relieved by high cAMP levels.

Expression of the cytochrome *c* gene *CYC1* is limited in the presence of glucose by the availability of the transcription factor HAP4, the synthesis of which is regulated at the transcriptional level. HAP4 binds to UAS2 of the *CYC1* promoter in a complex with HAP2 and HAP3 and is most likely responsible for the derepression of the gene in the absence of a fermentable carbon source (8). Induction of the gene in response to oxygen is controlled by different transcription factors, HAP1 and RC2 which bind to the adjacent UAS1 element (9).

Here we present an example in which a single transcription factor LAC9 responds to both the signal generated by the inducer and that generated by the presence of glucose. LAC9 regulates genes involved in lactose metabolism of the milk yeast *Kluyveromyces lactis*. Like the utilization of the disaccharide melibiose in *S. cerevisiae* ((10), see (11) for a review on the *S.cerevisiae* GAL regulon), lactose metabolism in *K. lactis* is closely linked to that of galactose and both sugars cause induction of gene expression (12−14). LAC9 is the *K. lactis* homolog of GAL4 as evident from the fact that it can complement a *gal4* mutation of *S. cerevisiae* (15) and vice versa (16). Both proteins bind to similar DNA sequences with the conserved motif 5′-CGG-N_5TN$_5$-CGG-3′ (17,18), and the amino acid sequence deduced from the cloned genes revealed three homologous domains one of which is involved in DNA binding (15,19).

The *LAC* genes of *K. lactis LAC12* and *LAC4*, encoding lactose permease and β-galactosidase, respectively (20,21), each contain two LAC9 binding sites in their 5′ non-coding region located between the divergently transcribed genes (18,22) our unpublished data). Deleting the *LAC9* gene not only eliminates transcriptional activation of the LAC and GAL genes but also eliminates the basal level of *LAC4* gene expression ((22) Gödecke et al., in preparation). Thus, LAC9 also activates transcription of *LAC4* in the absence of the inducer to about 1% of the fully induced levels.

LAC9 is synthesized constitutively and is able to bind to DNA when extracted from cells grown under non-inducing conditions (18,23). Genetic evidence suggests (24,25) that as in GAL4, the activating function may be regulated by a trans-acting negative factor which itself is inactivated by the inducer, probably a metabolite of galactose. Since LAC9 can interact with GAL80 (26) the negative regulator of *K. lactis* may function in a similar way.

Only in some of the natural *K. lactis* isolates are the *LAC* genes subject to glucose repression, and it has been shown recently that the transcriptional activator LAC9 is the target of the glucose

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signal (23). Depending on the allele of the *LAC9* gene, glucose has only a small and transient effect on *LAC4* induction (in *LAC9-1* strains) or reduces *LAC4* expression about 50-fold (in *LAC9-2* strains). The *LAC9-1* gene has been cloned and sequenced previously from strain ATCC Y1140 (15,19). Here we report on the cloning and analysis of the *LAC9-2* allele. We demonstrate that a glucose repressible *LAC9-2* strain can be converted to a glucose-insensitive phenotype both by a single nucleotide exchange in the DNA-binding domain of *LAC9-2* and by duplication of *LAC9-2*. We speculate that a negative factor present in glucose grown cells in limiting concentrations may interact with the DNA binding domain of the activator.

MATERIALS AND METHODS

K.lactis strains and yeast transformation

JA6 α *ade ura3 trp1* and its derivative DL9 α *ade trp1 ura3 lac9::URA3* have been described (18). DNA transformation was performed according to the method of Klebe et al. (27) with about 15 to 25μg of linearised DNA. When selection for Lac$^+$ was applied, cells were incubated in YEPD overnight at room temperature prior to plating.

Cloning of LAC9-2: To isolate the *LAC9-2* gene a plasmid pDL9Δ was targeted to the *LAC9* locus of strain JA6. pDL9Δ which contains *LAC9-1* sequences disrupted by the *URA3* gene was derived from pDL9 (18) by deleting a *Bam*HI-*Pvu*II-fragment of pBR322 vector sequences to remove one of the *Sal*I sites. This plasmid was cleaved with *Hpa*I 3′ to the *lac9::URA3* sequences and JA6 was transformed selecting for Ura$^+$ cells. In a stable transformant the expected structure of the *LAC9* locus with pDL9Δ integrated downstream of the *LAC9-2* gene was confirmed by Southern analysis.

Chromosomal DNA was isolated according to Sherman (28), purified with Quiagen 20 (Diagen, Düsseldorf), and digested with *Sal*I. 12μg of DNA were ligated at a concentration of 5μg/ml with 2 units of ligase overnight at 15°C. Upon transformation of *E. coli* 12 Amp^r colonies were obtained. Of the two that were tested both contained the *LAC9-2* gene linked to the pBR322 vector sequences of pDL9Δ. The resulting plasmid was named pLAC9-2.

S1 Mapping: To compare *LAC9-1* and *LAC9-2* sequences pLAC9-2 was cleaved with *Asp*718, 5′ end-labelled with [τ-^{32}P]ATP (29), cleaved with *Hin*dIII, and the two labelled fragments of 0.8 and 3.7 kb covering the 5′ and 3′ part of the *LAC9-2* gene, respectively, were isolated. Each of the fragments was mixed with 2μg of *Sal*I cut plasmid pJ432 containing the cloned *LAC9-1* gene (15), or with *pLAC9-2* as a control, denatured and hybridized in 50% formamide, 400mM NaCl, 40mM PIPES pH6.4, 1mM EDTA at 40°C overnight. S1 treatment was as described for RNA mapping (30) for 30 min at 37°C adding 300μl S1 buffer containing 600U/ml of S1. Following i-propanol precipitation the smaller fragment was analysed on a 6% sequencing gel and the larger fragment in a 1.2% agarose gel with 30mM NaOH (29).

DNA-sequencing: DNA-sequencing was performed by the method of Maxam and Gilbert (31) or after site-directed mutagenesis by the dideoxy chain-termination method (32) with the M13 sequencing primer (Boehringer, Mannheim).

Exchange of the Zn-finger between LAC9-1 and LAC9-2: The *Ava*II-fragment (position 684−2340 of (19) of pLAC9-2 was blunt-end ligated into a *Sma*I cleaved derivative of pUC12 (33) in which the *Acc*I site had been removed by cleavage with *Xba*I and *Hin*dII, fill-in and ligation. From the resulting clone pLAB92 the *Bgl*II-*Acc*I-fragment (position −42 to +370 of *LAC9-2*) which contained the two sequence alterations mentioned in the text was substituted by the corresponding fragment of *LAC9-1* giving pLAB91. The sequence of the *Bgl*II-*Acc*I fragment in both pLAB91 and pLAB92 was confirmed by Maxam Gilbert sequencing.

Oligonucleotide directed mutagenesis: To change codon 104 of the *LAC9-2* gene from TTG (Leu) to TGG (Trp) the pMa/pMc phasmid system of Stanssens et al. (34) was used. These two phasmids differ by a nonsense mutation in their ampicillin- (pMc) or chloramphenicol-(pMa) resistance genes, respectively. The 4.2 kb *Sal*I-*Hpa*I fragment of pLAC9-2 was inserted at the *Sal*I-*Sma*I sites of the two vectors giving plasmids pMa-D and pMc-D. A gapped duplex was formed between the single-stranded DNA of pMc-D and the *Kpn*I-*Bgl*II fragment of pMa-D creating a single-stranded region of 725 bp within the *LAC9-2* gene (position −42 to +683). The oligonucleotide 5′-GAAGAAGTGG-AAATGTTCC-3′ was annealed and the gap was filled with DNA polymerase I (Klenow fragment) as described (34). Plasmid DNA was isolated from a pool of 580 Amp^r/Cm^r transformants of the mismatch-repair deficient E.coli strain WK6mutS (35). To enrich for the progeny of the mutant DNA strand the plasmids were retransformed and Amp^r colonies were selected. Eight Amp^r/Cm^s trans-formants were then sequenced out of which four contained the expected mutation and were named pMa-D104.

Construction of LAC9 mutant strains: K. lactis strains containing the desired modifications of the chromosomal *LAC9* locus were constructed by one-step gene replacement (36,37) or integrative transformation. In each case strain JA6 or its derivatives were used as the recipient strains.

As described for JA6-1 (23) strains JA6-A12, JA6-A2 and JA6-104 were obtained by homologous recombination between *LAC9* sequences on *Eco*RI-*Pst*I cleaved plasmids pLAB91, pLAB92 and *Ava*II cleaved pMa-D104, respectively and the *lac9::URA3* locus in the disruption strain DL9 (18) resulting in Lac$^+$Ura$^-$ transformants.

To introduce a second *LAC9* gene, integrative vectors were constructed from pLAC9-2: the *S. cerevisiae URA3* gene of pBR322-ura (pBR322 plus the *URA3* gene on a 1.1 kb *Hin*dIII fragment) was inserted as a *Eco*RI-*Sal*I fragment upstream of *LAC9-2* to give pLI-2. The 5′end of the *LAC9-2* gene was exchanged against that of *LAC9-1* as a *Sal*I-*Kpn*I fragment to give pLI-1. The non-identity of pLI-1 and pLI-2 was confirmed by S1 treatment as described above.

These plasmids were integrated at the *LAC9* locus after cleavage with *Bst*EII 3′to the *LAC9* coding region. Insertion of pLI-1 in JA6 and JA6-1 resulted in JA6-2/1 and JA6-1/1 and insertion of pLI-2 in these strains gave JA6-2/2 and JA6-2/1, respectively.

Analysis of yeast DNA: Yeast chromosomal DNA was analysed by Southern blotting using Hybond membrane (Amersham Buchler, Braunschweig) and a non-radioactive labelling and detection kit (Boehringer, Mannheim) as described previously (23).

β-galactosidase assay: Cells were grown in yeast nitrogen base (YNB) containing all amino acids (28) and 2% glucose to mid-log phase, harvested and diluted to an OD_{600} of about 0.3 into fresh medium containing 2% glucose and 2% galactose. Cell extracts were prepared by breaking the cells with glass beads in a Braun homogenizer (Braun, Melsungen) as described (23). Protein concentration was determined according to Lowry (38) and β-galactosidase activity was measured using o-nitrophenyl-β-D-galactoside (30mM) as substrate. Activities were expressed as nmoles of substrate converted per min at 37°C and were determined in at least three independent experiments. Standard deviations were around 20%.

RESULTS

Cloning of the LAC9-2 gene

Induced β-galactosidase activities in *K. lactis* strain JA6 are decreased about 50-fold by the presence of glucose (23). In contrast, only a 2-fold reduction by glucose is seen with strain Y1140 (12) and with JA6-1, a strain isogenic to JA6, but harbouring the LAC9 gene of Y1140 (23). Hence the difference has to be due to the *LAC9* allele and we set out to isolate the *LAC9* gene of JA6 (*LAC9-2*). We used the cloned *LAC9-1* gene of strain Y1140 (15) for integrative cloning (39) following the strategy outlined in Figure 1. The integrative plasmid pDL9 carrying a disrupted *LAC9-1* sequence was targeted to the *LAC9-2* locus by cleavage 3′ to the *LAC9* coding region. In Ura^+Lac^+ transformants the *LAC9-2* gene and the disrupted *LAC9-1* gene were tandemly arranged separated by pBR322 vector sequences (Figure 1). By cleavage with *SalI* the intact *LAC9-2* gene linked to vector sequences could be obtained and cloned in *E. coli* (see Material and methods for details). The structure of plasmid pLAC9-2 is shown in Figure 1.

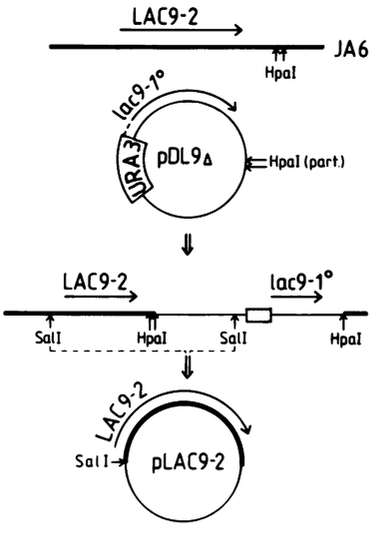

Figure 1: Strategy of isolation of the *LAC9-2* gene from strain JA6 by integrative cloning.
Plasmid pDL9Δ containing a disrupted *LAC9-1* gene (see Material and methods for details) was integrated 3′to the *LAC9-2* gene after transformation of JA6 with *HpaI* cleaved pDL9Δ plasmid DNA. Chromosomal DNA of the integrant was cut with *SalI*, ligated, and transformed into *E. coli* giving the Amp^r plasmid pLAC9-2.

LAC9-1 and LAC9-2 differ in the 5′ part of the coding region.

To avoid resequencing of the 2.6 kb gene we used nuclease S1 treatment of heteroduplexes to detect differences between the two alleles. Two labeled fragments covering the 5′ and 3′ part of the *LAC9-2* gene, respectively, were isolated and each was hybridized to an excess of linearized and denatured plasmid pJ-LAC9 (15) carrying the *LAC9-1* gene. Hybrids were treated with S1 and analyzed on denaturing gels. Whereas no cleavage was observed with the 3.7 kb fragment covering the 3′ part of the gene (data not shown), two labeled cleavage products were obtained with the 0.8 kb 5′ end (Figure 2, lanes c and d). These bands were not seen in a control hybridization using the homologous gene (lane e) and therefore should result from S1 cleavage of heteroduplexes. The stronger band of 722 bp corresponds in size to the endpoint of yeast sequences in the two clones (Figure 2 bottom) whereas the faint band indicated an S1-suseptible site about 440 bp from the label and about 240 bp downstream of the ATG initiation codon of LAC9.

Sequencing of a 409 bp fragment (position −42 to +367) covering the S1 cleavage site revealed two differences between the *LAC9-1* and *LAC9-2* sequences. The first is a 5′-AAC-3′ insertion in *LAC9-2* which extends a stretch of eight asparagine codons (codons 77 to 84) by one residue (Figure 3). The position of this three-nucleotide-loop in the heteroduplex is identical to that indicated by the S1 cleavage site. In addition, however, there was a second difference located in codon 103 of *LAC9-1:* a G-C to A-T transversion resulted in a Trp→Leu mutation. Since this one-base pair mismatch had not been cleaved by S1 under our initial assay conditions, additional differences between the two genes could not be excluded. We therefore examined if either of these mutations or both together were responsible for the glucose repressible phenotype of the *LAC9-2* strain JA6.

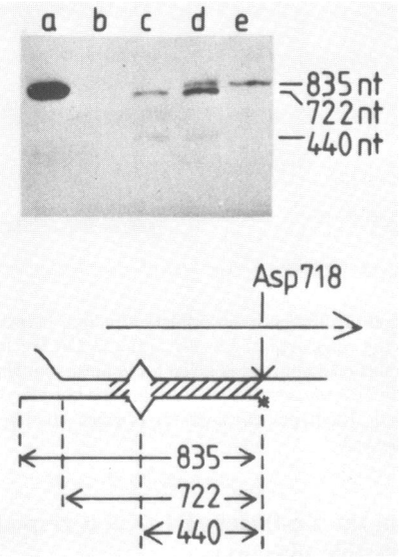

Figure 2: S1 treatment of *LAC9-1/LAC9-2* heteroduplex molecules.
An 835 bp fragment covering the 5′end of the *LAC9-2* gene was 5′end-labelled at the *Asp*718 site (position +683), isolated, denatured and hybridized to linearized denatured plasmids containing *LAC9-1* or *LAC9-2* sequences.
Top: Autoradiograph of the reaction products of S1 treated samples after gel electrophoresis. a) the labelled fragment without S1 treatment; b) the labelled fragment treated with 600 U/ml of S1; c) labelled fragment plus plasmid pJ432 containing the *LAC9-1* gene (15) treated with 600 U/ml of S1; d) same as c) but with 200 U/ml of S1; e) labelled fragment plus plasmid pLAC9-2. Bottom: Schematic representation of the heteroduplex formed between the *LAC9-2* fragment and the *LAC9-1* gene. The non-homologous ends are *LAC9* flanking sequences differing between the two plasmids. The hatched area is the *LAC9* coding region.

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pLAC9-1  GCC AAC AAC AAC AAC AAC AAC AAC AAC     AAG AAA TCA AGT GAA GTA
         76                                      85
         Ala Asn Asn Asn Asn Asn Asn Asn Asn Asn Lys Lys Ser Ser Glu Val
                                                 86
pLAC9-2  GCC AAC AAC AAC AAC AAC AAC AAC AAC AAC AAG AAA TCA AGT GAA GTA

pLAC9-1  ATG CAC CAG GCG TCG GAT GCT TGC AGG AAG AAG AAG TGG AAA TGT TCC
         91                                          Trp-103
         Met His Glu ALa Cys Asp Ala Cys Arg Lys Lys Lys     Lys Cys Ser
         92                                          Leu-104
pLAC9-2  ATG CAC CAG GCG TCG GAT GCT TGC AGG AAG AAG AAG TTG AAA TGT TCC
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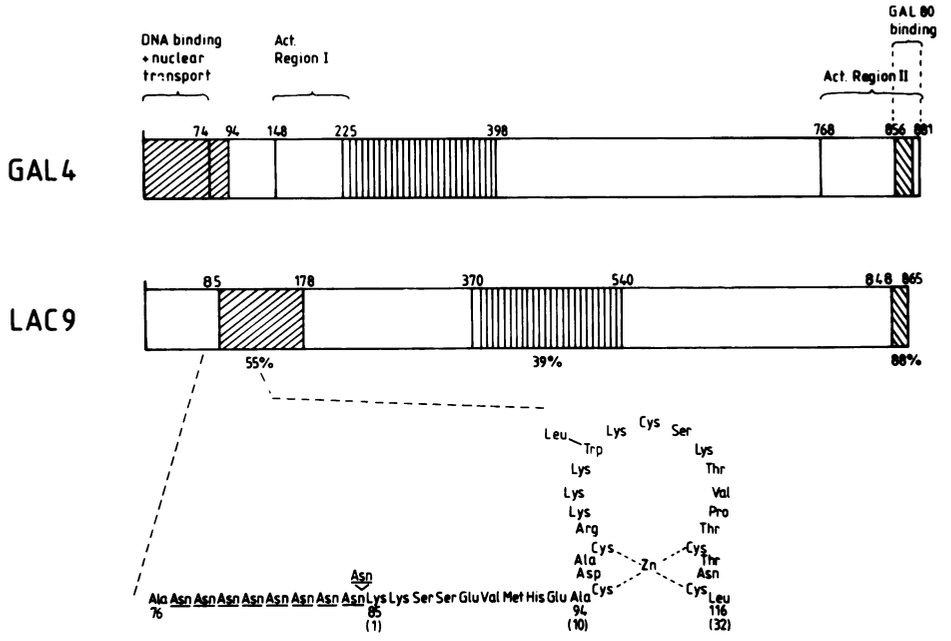

Figure 3: Location of differences in primary structure between *LAC9-1* and *LAC9-2*
Top: Protein coding sequences of *LAC9-1* (15) and *LAC9-2* from codon 76 to 106/107. Bottom: Location of the deviations within the LAC9 protein. The GAL4 protein with its functional domains is given for comparison. Hatched regions represent strings of amino acids with the indicated degree of identity (15,19). Numbers on top of the bars give the amino acids forming the borders of these domains. In the enlargement of a portion of *LAC9-1* the two sequence alterations of *LAC9-2* are again indicated. The hypothetical Zn-finger DNA-binding motif is shown. Numbering of amino acids is given for LAC9-1 and for the corresponding position in GAL4 (in brackets).

A mutation in the Zn-finger of LAC9 is responsible for the glucose-repressible phenotype.

First we introduced both mutations into *LAC9-2* by exchanging the 409 bp fragment we had sequenced (for details see Materials and methods). The resulting *LAC9-A12* gene had the *LAC9-1* sequence at the indicated positions in the *LAC9-2* allele. This *LAC9-A12* allele was used to transplace *lac9::URA3* in strain DL9, the lac9$^-$ derivative of JA6 (18). As a control a similar replacement was performed with the original *LAC9-2* allele leading to strain JA6-A2. β-Galactosidase measurements demonstrated that JA6-A2 as well as JA6 were glucose repressed whereas the new strain JA6-A12 is clearly inducible in the presence of glucose (Figure 4). Therefore, one or both mutations on the transplaced fragment had to be responsible for the *LAC9-1* phenotype.

To separate the two mutations we changed the leucine codon of *LAC9-2* (amino acid 104) to tryptophan by site-directed mutagenesis giving *LAC9-104*. Again this allele was introduced into DL9 by transplacement and the resulting strain was named JA6-104. As also shown in Figure 4 this strain behaved in almost exactly the same way as JA6-A12. We could conclude that the Trp-103 residue caused the glucose insensitive-phenotype of LAC9-1 whereas the insertion of the Asn codon had no significant effect.

Interestingly amino acid 103/104 is located in the Zn-finger which represents part of the DNA binding domain (40) of LAC9. This region is well conserved between LAC9 and GAL4 of *S. cerevisiae* (Figure 3 bottom and (41). GAL4 contains a leucine residue at the corresponding position of its Zn-finger, just like the repressible *LAC9-2* protein had been shown to confer glucose repression to *LAC4* gene expression when it was introduced into *K.lactis* substituting LAC9 function (16).

Slight overproduction of LAC9-2 overrides glucose repression

The dominance or recessiveness of the glucose repressible phenotype should give some clue to the potential mechanism. Since *K.lactis* is unstable as a diploid we decided to introduce two *LAC9* genes into a haploid cell: Using the otherwise isogenic

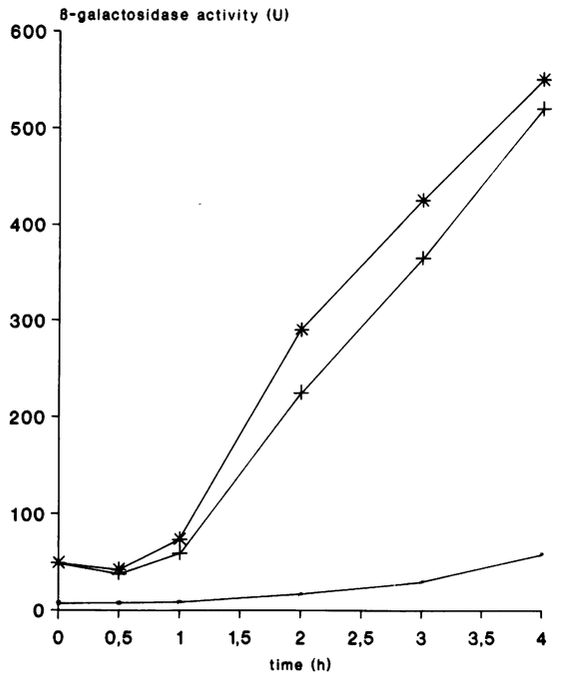

Figure 4: β-Galactosidase induction kinetics.
Strains JA6-A2 (·), JA6-A12 (*), and JA6-104 (+) containing the *LAC9-2* gene, the *LAC9-2* gene with the 5'segment of *LAC9-1*, and the *LAC9-2* gene with the Leu→Trp mutation of amino acid 104, respectively, were pregrown in glucose. They were diluted to an OD_{600} of 0.3 into fresh medium containing 2% glucose and 2% galactose at time zero. β-Galactosidase activities were measured in crude extracts prepared at the indicated times after addition of the inducer and are expressed in units per mg of protein.

strains JA6 and JA6-1 (23) harbouring *LAC9-2* and *LAC9-1*, respectively, as hosts partial diploid strains were constructed by integrating *LAC9-1* and *LAC9-2* containing plasmids giving the four possible combinations of two *LAC9* gene copies as outlined in Figure 5A. The Southern blot (Figure 5B) shows that not more than one plasmid copy had been integrated since the intensity of the *Hin*dIII fragment of 2.7 kb and 3.1 kb resulting from plasmid and flanking sequences, respectively, were about the same.

β-Galactosidase activity was followed after addition of galactose to glucose-grown cells (Figure 5C). In all strains containing two *LAC9* genes (only three of the four possible combinations are shown) there was no glucose repression. The effect of glucose was even less than in JA6-1, the so-called non-repressible strain which showed a slightly longer delay at the onset of induction and clearly lower induced enzyme levels. Surprisingly, there was no difference in the kinetics of induction between strains having two *LAC9-2* (JA6-2/2) or two *LAC9-1* genes (JA6-1/1) but the latter one showed elevated level of β-galactosidase throughout the time course. JA6-2/2 was identical to strains JA6-1/2 (not shown) and JA6-2/1, leading to the conclusion that an increase in gene dosage from one to two is sufficient to override glucose repression and the glucose insensitive allele had no additional effect under conditions of LAC9 overproduction.

Strains containing two *LAC9* genes also had higher basal levels of *LAC4* gene expression. Under repressed conditions strain JA6-2/2 gave 5−10 fold higher β-galactosidase activities than JA6. In JA6-1/1 the basal level was about 60% of the normal induced level. These data indicate that *LAC4* gene expression is limited by the amount of active LAC9 protein both in the presence and in the absence of inducer. As pointed out earlier the *LAC9-1* allele present in a single copy also results in higher enzyme activities on glucose (23).Thus, in all strains there is a correlation between the glucose insensitive phenotype and an elevated level of β-galactosidase in glucose grown cells suggesting that glucose acts by negatively affecting the activity of the transcriptional activator LAC9.

DISCUSSION

We have cloned and analysed *LAC9-2*, a second allele of the gene encoding the transcriptional activator LAC9. The product of this allele confers sensitivity to glucose. Two differences to the previously cloned *LAC9-1* allele (15) were detected, a 5'-AAC-3' insertion and a G→T transversion . The latter is responsible for the phenotype as shown by site-directed mutagenesis and changes amino acid 103 of LAC9-1 (104 of *LAC9-2*) from tryptophan to leucine. The 3 bp insertion does not seem to affect the properties of LAC9 that were tested here. It extends a stretch of eight asparagines (aminoacids 77 to 84 of *LAC9-1*) by one and might be a consequence of slippage by the replication machinery on this stretch of AAC-codons. Using S1 nuclease search for allelic differences, the relevant mutation was initially detected only fortuitously by being linked to a 3 bp insertion. However, later on we were able to cleave the single mismatched basepair using higher concentrations of S1.

The mutation conferring glucose sensitivity is located within the Zn-finger sequence motif highly conserved between LAC9 and GAL4 (15,19) that has been shown to be involved in DNA binding in both proteins (40,42). The Trp→Leu exchange increases the homology to GAL4 which also has a Leu-codon at the corresponding position. As a consequence within a stretch of 14 residues 13 are identical between LAC9-2 and GAl4. Interestingly, GAL4 also confers glucose repression to the LAC genes when introduced into *K. lactis* (16). Therefore, the mechanism of glucose repression of the GAL genes which involves the GAL4 protein is probably similar in *S. cerevisiae* and *K. lactis*. However, in *S. cerevisiae* additional GAL4 independent pathways of repressing the GAL genes exist (43−45) which might explain why glucose insensitive variants of GAL4 have escaped detection until now.

Not only strains containing the *LAC9-1* allele are non-repressible by glucose but also those containing a duplication of the *LAC9 gene*, including the one with two copies of *LAC9-2* encoding the glucose sensitive activator. By quantitating LAC9 binding activity in crude cell extracts of JA6-2/2 we have observed a 2 and 4−5 fold higher activity in glucose- or galactose-grown cells, respectively (Zachariae and Breunig, in preparation). Thus, a moderate overproduction of the activator is sufficient to overcome the inhibiting effect of glucose. The elevated β-galactosidase levels in these strains indicate that *LAC9* activity is rate limiting for *LAC4* gene expression under all growth conditions. The higher basal levels of enzyme activity in the haploid strains JA6-1 (23) and JA6-A12 as compared to JA6 or JA6-A2 suggests that the LAC9-1 protein present in these strains has a higher activity. The correlation of elevated basal levels of *LAC4* expression with a glucose-insensitive phenotype further suggests that glucose acts by reducing the activity of LAC9. LAC9-1 would then be a variant which is less sensitive to the inhibitory effect of glucose.

How could the glucose signal influence the activity of the activator? In principle its potential to activate transcription, its

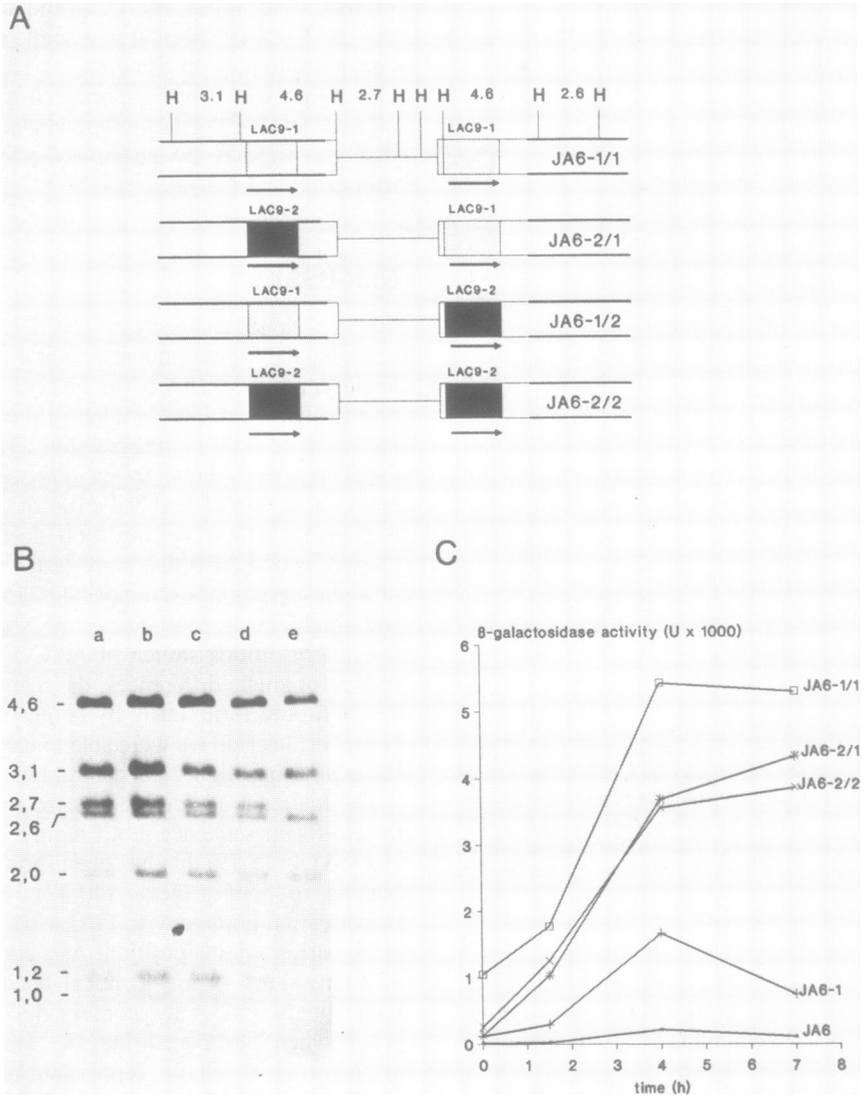

Figure 5: β-galactosidase induction in partial diploid strains.
A Schematic representation of the chromosomal *LAC9* locus in partial diploid strains. Double lines indicate yeast sequences of host (thick lines) and plasmid origin (thin lines), respectively.The single line represents pBR322 vector sequences. The *Hin*dIII restriction sites (H) and lengths of fragments are indicated. The order and alleles of the *LAC9* gene copies (filled boxes) give the names of the strains. B Southern blot of *Hin*dIII digested DNA of strain JA6-1/2 (a), JA6-2/2 (b); JA6-2/1 (c), JA6-1/1 (d) and JA6 (e). The blot was probed with plasmid pJ-LAC9 (15). C β-galactosidase activities for strains JA6-1/1 (□); JA6-2/1 (*) JA6-2/2 (×); JA6-1 (+) and JA6 (·) after addition of 2% galactose to glucose grown cells (compare Leg. to Fig.4).

ability to bind to DNA or both activities could be affected. At present we cannot rule out any of these possibilities. However, based on the finding that a point-mutation in the Zn-finger renders the protein largely insensitive to glucose we would like to suggest that the DNA-binding rather than the activating function of LAC9 is affected by glucose. In keeping with this proposal it has been observed that in *S. cerevisiae* cells the characteristic GAL4-dependent in vivo footprint is absent in glucose grown cells (46−48) indicating that binding of the activator is affected by glucose.

Regulation of DNA binding could occur in two ways:

First, in glucose the LAC9 target site could be inaccessible e.g because another protein binds to the same region. In the glucose-insensitive strains LAC9 could then serve as a better competitor either by having a higher affinity for DNA or by being present in higher concentrations. Although a *gal4* mutant in which the Leu-19 corresponding to Leu-104 in *LAC9-2* was converted to a proline indeed had drastically reduced binding activity (49) preliminary results did not reveal any differences in DNA affinity between LAC9-1 and LAC9-2 (unpublished observations). In addition, a protein binding to DNA with about the same affinity as LAC9 should be detectable in our in vitro binding studies and we do not have any evidence for that. In particular in a strain lacking LAC9 no footprint is seen (18). Also the in vivo studies in *S. cerevisiae* (47,48) do not give any indication that another protein is replacing GAL4 in glucose-grown cells. The protein involved in chromatin organization which binds to the same region does not seem to be regulated nor does it prevent GAL4 from binding (50). We therefore consider this first model unlikely.

In the second model the LAC9 protein itself is modified in glucose. This could either occur by a regulated post-translational modification or by complex formation with a protein or metabolite generated in the presence of glucose. Covalent modification has

been reported for GAL4 (51). GAL4 gets phosphorylated and at least one phosphorylated form is correlated with its highly active state. Upon addition of glucose this form disappears. However, it has not been shown yet that dephosphorylation is responsible for inactivation. If phosphorylation were to account for regulation of LAC9 activity by glucose, then LAC9-1 should be phosphorylated at least to the same extent as LAC9-2, since it is as active, but should be more resistant to dephosphorylation. In the overproducing strains a higher level of the phosphorylated highly active form might account for the glucose-insensitive phenotype although the idea that a phosphatase activity is limiting is not very likely. The same argument applies to a metabolite accumulating in glucose grown-cells.

In contrast, a protein synthesized or activated in response to the glucose signal could be present at limiting concentrations accounting for the 'titration' effect seen in LAC9 overproducing strains. LAC9-1 would then have a lower affinity for such a hypothetical 'glucose repressor'. This protein could bind to LAC9 preventing it from binding to DNA or it could bind to the LAC9/DNA complex destabilizing it or preventing activation of transcription. By comparing LAC9-2 DNA binding activity in extracts from glucose and galactose grown cells we observed a difference of only 2–3 (Zachariae and Breunig, unpublished). However, the postulated complex might dissociate under our conditions of extract preparation (18) and not be able to reform with the DNA-free LAC9 protein. Alternatively the activity of the repressor might be regulated by phosphorylation. Since we have a strong phosphatase activity in our extracts such a modification may be lost.

The regulation of DNA binding by glucose as proposed for LAC9 is opposed to the effect of lactose and galactose which seem to regulate the transcription activating function. Recently Kim and Guarente (52) suggested that the Zn-finger of the transcriptional activator HAP1 is not only involved in DNA binding but also in transcriptional activation. It may turn out to be more generally true that these two functions are not as independent as current models imply. Further analysis of the two forms of LAC9 should allow to determine whether this activator is really composed of two separate domains which are independently regulated or whether both functions are structurally linked.

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